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Non-technical write-up of summer research for the Department of Homeland Security

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Native chemical ligation (NCL) occurs by the formation of an amide bond between two polypeptide fragments, one of which contains a C-terminal thioester and the other an N-terminal cysteine. This process enables incorporation of unnatural amino acids into proteins and other forms of protein engineering. Protein engineering is important to development and discovery of therapeutics and improving understanding of biological processes. NCL has also been used for fabrication of protein microarrays that may be useful for development of bioassay methodology and are directly applicable to detection of biological weapons. For these reasons, NCL is regarded as one of the most important and useful tools available to chemists and biochemists for investigation of biological systems.

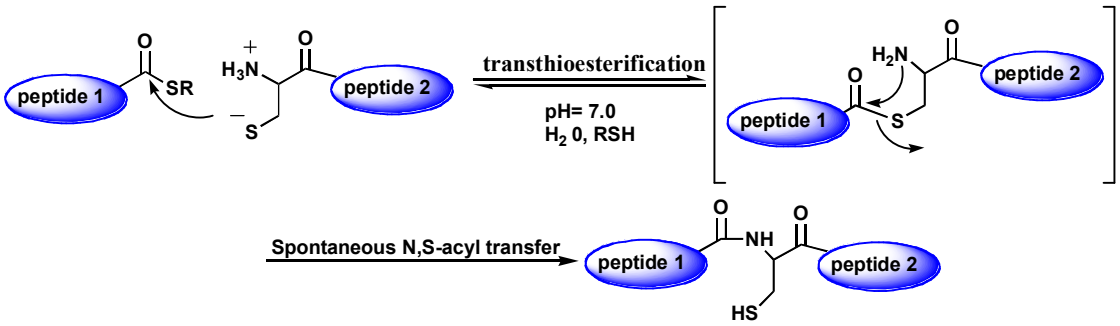

Figure 1. Creation of amide bond by NCL between two polypeptides

As seen in Figure 1, NCL relies on the availability of C-terminal polypeptide α-thioesters. These molecules can only be prepared by chemical synthesis or using recombinant techniques. The most successful approach to synthesis of polypeptides employs solid phase peptide synthesis (SPPS) methodology. Two approaches to SPPS are commonly employed. The first, termed Boc methodology, employs Boc protecting groups on the nascent polypeptide during the SPPS. Final cleavage of the formed polypeptide from the solid support (resin) requires the use of highly toxic hydrogen fluoride. The second SPPS method, termed Fmoc/*t*-Bu methodology, uses the much milder trifluoroacetic acid to cleave the polypeptide from the resin. Hence, Fmoc/*t*-Bu peptide syntheses are highly preferable to Boc syntheses.

However, C-terminal peptide α-thioesters are not stable to the relatively nucleophilic base piperidine which is employed in Fmoc/*t*-Bu syntheses. Therefore, the development of methods that allow Fmoc/*t*-Bu SPPS methodology to be applied to the synthesis of C-terminal polypeptide α-thioesters is essential to researchers who wish to employ the powerful technique of NCL.

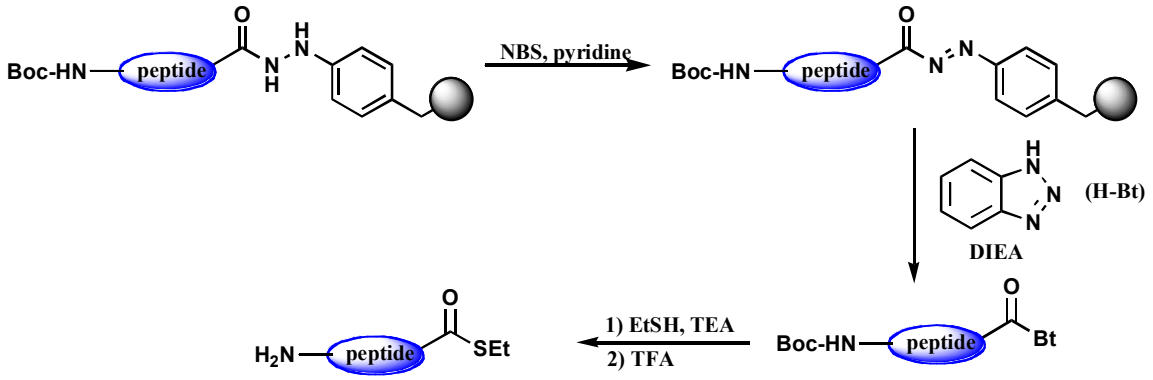

Figure 2. Proposed synthetic methodology for preparation of C-terminal peptide thioesters

My project at LLNL this past summer was to improve upon the available methodology for synthesis of C-terminal polypeptide α-thioesters (all of which methods suffer from certain disadvantages requiring too much detail to discuss herein). Our initial approach to synthesis of α-thioesters is outlined in Figure 2. The approach utilizes a resin containing an aryl hydrazine linker to which the growing polypeptide chain is attached. The aryl hydrazine linker can be oxidized under mild conditions to the corresponding diazene. Our objective was to use the weak N-nucleophile benzotriazole to cleave the peptide from the resin. The acyl benzotriazole formed by the cleavage may be thiolyzed using ethanethiol and triethylamine to form the corresponding C-terminal polypeptide α-thioester, which can then be employed in NCL.

My initial experiments failed to result in formation of any α-thioester. Instead, the exclusive product of acyl diazene cleavage was the peptide hydrolysis product. A number of experiments were performed to identify the stage at which hydrolysis was occurring. It was found that hydrolysis occurred during the benzotriazole-mediated cleavage of the acyl diazene. After extensive experimentation, I discovered that C-terminal polypeptide α-thioesters could, in fact, be formed by performing the acyl diazene cleavage in the absence of diisopropylethylamine (DIEA).

I performed other experiments to study the variables that could improve the ratio of the hydrolysis product to the thiolysis product and was able to obtain replicable results in which the product mixture was 30% α-thioester and 70% hydrolysis. While the yield must still be improved for this to represent a viable method of peptide α-thioester synthesis, it does represent significant progress towards development of such a method. I was able to effect a three- to five-fold improvement in the yield of α-thioester relative to the α-thioester yield when ethanethiol was used to cleave the acyl diazene, a promising result which merits further investigation.

I performed some experiments utilizing alternative N-nucleophiles such as imidazole to cleave the acyl diazene, as benzotriazole appears to compete poorly

with water in the cleavage reaction. Some promising results were obtained, suggesting that use of a slightly stronger N-nucleophile may increase the yield of C-terminal polypeptide α-thioester.